Original Article

The mutation of the rdxA gene in metronidazole-resistant *Helicobacter pylori* clinical isolates

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Abstract

Backgrounds: Antibiotic resistance is an increasing problem throughout the developed world, and knowledge about different resistance mechanisms is consequential for efficient treatment of bacterial infections. Although metronidazole has been frequently used in treatment regimens for *H. pylori* infection, but antibiotic resistance is now a major contributing factor in treatment failure. Nevertheless metronidazole has been greatly used as a critical component of combination therapies for *H. pylori* infection.

Objective: This study is trying to describe the mutational mechanisms of metronidazole resistance in *H. pylori* in our clinical isolates in Isfahanian patients, Iran and compare with the findings of previous studies in world.

Materials and Methods: MIC values of metronidazole for *H. pylori* strains were determined by E- test. Both rdxA and glmM genes used for confirmation of isolates as *H. pylori* and then amplification of another rdxA oligonucleotide pair was done. Finally, the six resistant strains were sent to sequencing for other processing and further analysis was done by software.

Results: The result of six clinical isolates in comparison with 26695, J99 and 69A as a sensitive and resistant reference strains showed plenty of mutations. No frame shift and nonsense mutation was seen in our clinical isolates.

Conclusion: An interesting finding in metronidazole-resistant strains in our study was the detection of one mutation not previously described in the literature in the rdxA gene and this W(209)R substitution presumably plays a role in inducing metronidazole resistance.

Key Words: *Helicobacter pylori*, metronidazole, rdxA gene, resistance

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Received: 08.05.2013, **Accepted:** 06.08.2013

Access this article online	
Quick Response Code:	Website: www.advbiores.net
	DOI: 10.4103/2277-9175.128469

INTRODUCTION

Antibiotic resistance is an increasing problem throughout the developed world, and knowledge about different resistance mechanisms is consequential for efficient treatment of bacterial infections. One important class of antibiotics, the 5-nitroimidazole (5-Ni) drug derivatives, includes metronidazole [1-(2-hydroxyethyl)-2-methyl-5-nitroimidazole].[1]

How to cite this article: Mirzaei N, Poursina F, Moghim S, Rahimi E, Safaei HG. The mutation of the rdxA gene in metronidazole-resistant Helicobacter pylori clinical isolates. Adv Biomed Res 2014;3:90.

Metronidazole (Mtz) has been greatly used as a critical component of combination therapies for *H. pylori* infection.[2] Metronidazole, a synthetic nitroimidazole, is a prodrug and becomes active when reduced in the cytosol of the microorganism to a toxic metabolite. Unstable metronidazole radicals react quickly with proteins, RNA and DNA, ultimately resulting in cell death.[3,4] Although there have been conflicting reports concerning the clinical impact of metronidazole resistance in *H. pylori*, many studies have proven that resistance to this class of antibiotics does diminish the effectiveness of metronidazole-containing eradication regimens.[5] Nevertheless, treatment regimens for the eradication of *H. pylori* according to new data still include metronidazole in combination with other agents.[6] Ergo, the precise function of genomic variants leading to the change in nitroreductase remains slightly controversial when accounting for the formation of metronidazole resistance in *H. pylori*.[7]

Goodwin *et al*. first proved that the major mechanism of metronidazole resistance in *H. pylori* was due to null mutations in the rdxA gene (H0954 in the *H. pylori* genome database), which encodes an oxygen-insensitive NADPH nitroreductase whose expression is necessary for intracellular activation of the drug and generate a protein of 210 amino acids.[7] The ability of rdxA gene inactivation to confer resistance has recently been confirmed by Moore and Salama (2005) by saturation transposon mutagenesis of the *H. pylori* genome.[8]

Introduction of metronidazole resistance by rdxA knockout mutagenesis and the virtual absence of rdxA mRNA levels or of RdxA protein expression in metronidazole-resistant strains provides further evidence of the importance of this gene in the resistance mechanism.[9] Nonetheless, a number of resistant strains have been reported in which the rdxA gene appears unaltered. Various features of *H. pylori* RdxA, such as the presence of multiple cysteine residues and alkaline pI, may contribute to both a lower redox potential and greater substrate specificity for metronidazole.[5] Metronidazole and related nitroimidazole compounds represent momentous factors in *H. pylori* eradication regimens. The prevalence of resistant isolates in patients varies depending on the geographical region in question, but has reached alarming proportion in both developed and developing nations. Thereupon, in regard to high resistance rate to metronidazole, a necessity for local studies is felt. This study is trying to describe the mutational mechanisms of metronidazole resistance in *H. pylori* in our clinical isolates in Isfahanian patients, Iran and compare with the findings of previous studies in world.

MATERIALS AND METHODS

Bacteria and growth conditions

Among 110 patients who referred to the endoscopy unit at Al-Zahra hospital, Isfahan, Iran in 2011, no one did exposure to any prior antibiotics for *H. pylori* infection in the past 2 weeks. In addition, written informed consent was obtained from each subject to participate in the study. The biopsy specimens were placed on the brucella agar (Merck, Germany) supplemented with 5% defibrinated sheep blood (Bahar afshan, Iran), L-cystein 2% (Merck, Germany), 10% fetal bovine serum (Sigma, USA), campylobacter selective supplement (Merck, Germany), and 5 mg/l of amphotericin B (Merck, Germany).[10] The plates were incubated into a microaerophilic atmosphere (10% CO_2, 6%O_2, and 84%N_2) by using of MART system (Anoxomat, Lichtenvoorde, the Netherlands) and relative humidity at 37°C for 3 to 5 days.

Determination of MIC

MIC values of metronidazole for *H. pylori* strains were determined by the epsilometer test (E-test, Biomerieux, France). A suspension equal to the McFarland tube no. 3 was prepared for each isolate. The E-test strips for the antibiotics were aseptically placed onto the dried surface of inoculated agar plates (Mueller Hinton agar, Merck, Germany) and sheep blood 5% v/v (Bahar afshan, Iran), according to CLSI instruction. The plates were incubated under microaerophilic condition at 35°C for 72 hours or longer until a visible inhibition ellipse is seen. Isolates were considered resistant to metronidazole if the MIC was ≥ 8 μg/ml. Also, the reference strain was cultured and reference strain in this study was *H. pylori* 26695.

Confirmation of isolates with glmM and rdxA genes

The ureC gene encodes for a phosphoglucosamine mutase; this gene is irrelevant to urease production, so it was renamed glmM. This gene is considered a "housekeeping" gene, and it participates directly in cell wall synthesis.[9,11] We designed two pair of oligonucleotide primers, one of them for assuring of undoubted existing *H. pylori* in biopsies (glmM) and another one for confirming the presence of the rdxA gene in bacterium [Table 1]. DNA was extracted from all resistant *H. pylori* isolates (27 strains) and reference strain 26695 by QIAamp tissue kit (Qiagen, Germany) according to the manufacturer's instruction.

MEGA4 multiple alignment tool and Allele ID version 6 software were used for alignment and primer designing, respectively. MEGA4 was used to align all glmM and rdxA sequences of *H. pylori* that were in NCBI database and identify conserved areas for the primer design. By using primer blast tool, primers

Table 1: Primers used in this study

Primer pair	Encoded protein	Nucleotide sequence (5 → 3)	Size
glmM-F glmM-R	Phosphoglucose amine mutase	5- TGCTTGCTTTCTAACACTAACG 5- TTGATGGCGATGCTGATAGG	355 bp
rdxA-F rdxA-R	Oxygen insensitive NADPH nitroreductase	5-GCAACTATCCAATCCCATCAAG 5-GCCAGACTATCGCCAAGC	360 bp
rdxA-F rdxA-R		5-GCAGGAGCATCAGATAGTTCT 5-GGGATTTTATTGTATGCTACAA	886 bp

were controlled for confirmation and thermodynamics characteristics were assessed by Gene runner software.

The expected PCR product for glmM and rdxA was 355 bp and 360 bp, respectively. The cycling program for both genes was 1 cycle at 95°C for 10 minutes; 30 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds and a final elongation step at 72°C for 1 minute.

PCR amplification of rdxA

Size of the rdxA gene sequence is 633 bp. Therefore, other pair of oligonucleotide primer for rdxA is recommended. So, a set of primer with size of 886 bp is chosen which detects whole sequence of the rdxA gene, while the first rdxA primer (360 bp) was designed just for confirming the presence of the rdxA gene in bacterium. The cycling program was 1 cycle at 94°C for 2 minutes; 30 cycles of 94°C for 40 seconds, 50°C for 40 seconds, and 72°C for 1 minute; and a final elongation step at 72°C for 10 minutes. The expected PCR product was a single 886 bp band for the rdxA gene.[7,12] The PCR products were ran on 1% agarose gel (Invitrogen, USA) in TBE buffer (Merck, Germany). Among resistant clinical isolates, six strains were sent to sequencing for other processing.

DNA sequence determination and analysis

PCR products were purified and sequenced by Bioneer Korea Company. The DNA sequence analysis and comparisons or similarity were done by using the software (BLASTX network) available over the Internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) and clustalW2 software (simGene.com) for alignment. The mutations of rdxA sequencing were compared with the metronidazole susceptible ATCC 26695 (AE000511.1), J99 (AE001439.1) and metronidazole resistant strain 69A (AF315501.1).

Nucleotide sequence accession numbers

The nucleotide sequences referred to in this paper are available in GenBank under accession numbers KC491779-KC491784.

RESULT

Forty-eight *H. pylori* isolates without history of anti-*H. pylori* therapies were obtained from 110 patients biopsy samples referred to the endoscopy unit at Al-Zahra hospital in Isfahan, Iran. The MICs of all isolates were tested by E-test. Metronidazole resistance was proven in 27 of 48 patients. As expected, 26695 strain was sensitive to metronidazole. Among metronidazole-resistant clinical isolates, 26.3% (5 cases) were males and 75.9% (22 cases) were females. In general, frequency of resistance rates of metronidazole was 56.3%. In the 27 metronidazole-resistant strains, 10 isolates had MICs equal or more than 64 μg/ml and the remaining 17 isolates had a MIC in the range of 8-32 μg/ml.

As it is mentioned above, six resistant clinical isolates to metronidazole were sent for sequencing and the results of that are listed in Table 2. In the present study, alterations were approximately observed in all resistant clinical isolates except sixth patient. Firstly, analysis was compared with 26695 as a reference strain. At one glance, it is conspicuous that there are seven discrepancies between amino-acid structure of 26695 and 69A as a resistant reference strain according to alignment program and the remaining structure is the same in both reference strains, while there are nine differences between amino-acid structure of 26695 and J99 as a sensitive strains to metronidazole. Four replacements in positions of 59, 97,131 and 172 are similar in J99 and 69A in contrast with 26695 [Table 2]. Conversion of D(59)N was seen in approximately all clinical isolates and reference strain J99 and 69A, too. Substitution of Q(6)H, R(131)H and V(204)I was seen in clinical isolates three times, while substitution of R(16)H, T(31) E and R(90)K was seen twice in first and second patients. Notwithstanding sixth patient had highest MIC among our metronidazole-resistant clinical isolates (256 μg/ml), no changes were seen as opposed to 26695 strain. It is supposed that other mechanisms have a main part in this resistant isolate.

In accord with Table 2, there is a new substitution W(209)R in fourth patient with relative high MIC (128 μg/ml) and position of this conversion is shown in Figure 1. Besides to missense mutations, plenty of silent mutations were observed. According to the result of the aligned sequences 15, 13, 14, 16 and 17 silent replacements were seen in first, second, third, fourth and fifth isolates in comparison with 26695, respectively. It is remarkable that there is no silent alteration in sixth patient unlike to its high MIC level. The following conclusion can be drawn from the aligned sequences is that there is 24 and 17 silent substitution in J99 as a sensitive strain and 69A as a resistant strain in contrast with 26695, respectively.

Table 2: Deduced RdxA amino-acid changes in metronidazole-resistant *H. pylori* isolates in comparison with 26695 strain

Strain	MIC	Amino-acid position and change in RdxA																					
	μg/ml	6	16	18	25	31	56	59	64	68	90	97	98	106	108	111	118	131	172	183	197	204	209
26695	S	Q	R	S	H	T	M	D	K	A	R	H	G	P	S	V	A	R	V	A	Q	V	W
J99	S	H	.[a]	.	.	.	.	N	.	.	K	T	S	.	.	.	.	K	I	V	K	.	.
69A	R	.	.	.	.	E	.	N	N	.	.	T	.	S	.	.	.	K	I	.	.	.	.
P-1	192	.	H	.	.	E	.	N	.	.	K	Y	.	.	.	.	.	.	.	.	.	.	.
P-2	128	H	H	.	.	E	.	N	.	T	K	.	.	.	.	.	T	K	.	.	.	I	.
P-3	192	H	.	F	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	I	.
P-4	128	H	C	.	R	.	.	N	N	V	.	T	.	S	A	.	.	K	.	.	.	.	R[b]
P-5	192	.	.	.	.	.	I	N	.	.	.	.	.	.	.	A	T	K	.	.	.	I	.
P-6	256	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Abbreviations: MIC, minimum inhibitory concentration; MTZ, metronidazole.
[a]The amino acid is the same as that of the 26695 strain (AE000511.1)
[b]A missense mutation resulted in the amino-acid substitution.

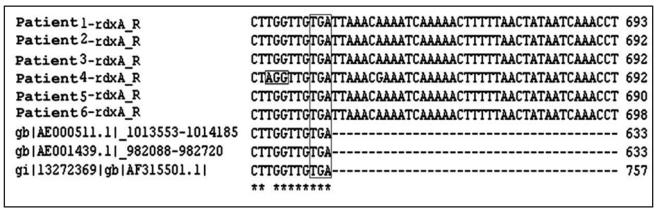

Figure 1: Position of new substitution in our study (P-4)

Alignment results clearly highlights that a sensitive strain 26695 and a resistant strain 69A have 95% similarity, while there is 94% resemblance between J99 and 69A. As it is mentioned before, the reason of existing 1% discrepancy is having more alterations in J99. On the other hand, there is 93% sameness between 26695 and J99.

DISCUSSION

H. pylori has grown a high degree of resistance to metronidazole, particularly in developing countries. Determination of antimicrobial susceptibility is therefore prominent, specifically when treatment has failed.[2] Recent studies of fresh clinical isolates indicated that metronidazole resistance frequently results from point mutation in rdxA (HP0954, in the fully sequenced genome of strain 26695), a gene that encodes an oxygen-insensitive NADPH nitroreductase. However, some researchers have questioned the generality of this interpretation.[13] More importantly, expression of RdxA protein is lower in metronidazole-resistant strains, so a potential role for rdxA in metronidazole resistance cannot be excluded.[9]

Debets-Ossenkopp *et al*. showed that the 200 bp deletion in the rdxA gene was a major factor in metronidazole resistance. In contrast, Kato *et al*. reported that there was no deletion of 200 bp in the rdxA gene in metronidazole-resistant strains.[14] From the other point of view, three surveys were done in Iran by Mohammadi (2005), Kargar (2010) and Abdollahi (2011) in Tehran, Jahrom and Kerman. These studies reported that some of the metronidazole-resistant isolates had 200 bp deletion in the rdxA gene while none of the sensitive isolates had this deletion.[14-16] In the present study, no deletion of 200 bp was seen in resistant *H. pylori* clinical isolates which was in accordance with Kato *et al*. research.[14] The investigations provided a large number of point mutations in the rdxA gene from metronidazole-resistant *H. pylori*, but the positions were not fixed.[2] Though, some of researchers believe that mutation causing Arg(16)His substitutions reported previously could be critical to the metronidazole-resistant phenotype. In agreement with these studies,[7-9,12,17-20] this cardinal mutation was seen in first and second isolates with MIC of 192 and 128 μg/ml, respectively. Substitution mutations in notable structural or catalytic domains of rdxA may lead to functional inactivation of nitroreductase.[20]

Also, Solca *et al*. had reported amino acid changes such as Arg(16)His, Arg(16)Cys, His(25)Arg, Asn(59)Asp, and Val(204)Ile. According to their research, these changes were seen just in resistant isolates[21] and these substitutions are in accordance with our study in resistant isolates with MIC ≥ 128 μg/ml. Known *et al*. examined the role of some other substitution which made strains resistant in North American isolates. Just two mutations such as Val(204)Ile and Arg(90)Lys[20] were similar in their and our research. In the majority of resistant strains, mutational inactivation of the rdxA gene prevents production of the protein or results in production of an abnormal polypeptide which is subsequently degraded.[22]

Bereswill *et al*. reported some amino-acid substitution in RdxA proteins in positions of 6 to 178 in Germany isolates.[23] In accordance with their studies, some of those substitutions such as positions of 6, 16, 31, 59, 90, 97, 118, and 131 are seen in our research. But conversion of Met(56)Ile and Ala(68)Val was more considerable, because of occurring lonely in resistant isolates. Recent studies revealed the RdxA amino-acid changes of Arg(10)Lys, Arg(16)His, Met(21)Ala, His(53) Arg, Met(56)Ile, Leu(62)Val, Ala(68)Val, Gly(98)Ser, Gly(163)Asp, and Ala(206)Thr in metronidazole-resistant strains, suggesting that those substitutions might be important in metronidazole resistance.[19] Some of these replacements such as positions of 16, 56 and 68 were observed in our metronidazole-resistant isolates. The finding that rdxA sequence variations detected in high-level metronidazole-resistant isolates did not cluster in a defined gene region limits the use of rdxA as a marker gene for metronidazole resistance.[23]

Among clinical isolates, solely one isolate was devoid of mutations on the rdxA. It is noteworthy to know that this isolate had the highest amount of MIC equal to 256 μg/ml among isolates. There are lots of mechanisms for acquisition of metronidazole resistance such as rdxA mutations, while the precise role of other genes for inducing the metronidazole resistance by *H. pylori* is unclear. Meanwhile, there is evidence that the metronidazole-resistant phenotype may arise in *H. pylori* without mutations in rdxA, suggesting the presence of additional metronidazole resistance mechanisms.[2,8] An interesting finding in metronidazole-resistant strains in our study was the detection of one mutation not previously described in the literature in the rdxA gene and this W(209) R substitution presumably plays a role in inducing a metronidazole-resistant isolate. In addition, all of these alterations are the first report in our country, Iran. One major advantage of using this approach is that it would recognize all resistant strains that carry mutations affecting expression of the rdxA gene, containing those that have not yet been identified by nucleotide sequence analysis.

Results from this research demonstrate that the patterns of gene mutation in the rdxA gene, both 26695 and J99 as a sensitive and 69A as a resistant strain, were diversified and we should have more information about our own region in order to prevent from high rate of resistance.

CONCLUSION

In conclusion, mutations in the rdxA gene were significantly associated with resistance to metronidazole, but as the number of strains in the present study was relatively small compared with other reports, further investigations with a large number of strains are required. Future work from our group will focus on the identification of other mechanisms responsible for metronidazole resistance phenotype.

ACKNOWLEDGEMENTS

This study was supported by Vice Chancellor of Research and Department of Microbiology, Faculty of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

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Source of Support: Isfahan University of Medical Sciences, **Conflict of Interest:** None declared.